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Optimising the ratio of long- to short-chain triglycerides of the lipid phase to enhance physical stability and bioaccessibility of lycopene-loaded beverage emulsions

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Summary

Oil-in-water beverage emulsions (pH3.2) with different long- to short-chain triglyceride (LCT to SCT) ratios were used to encapsulate lycopene. Beverages containing 3% w/w oil from carrier lipids were prepared as follows (w/w): 100:0, 75:25, 50:50, 25:75 and 0:100 (corn oil:tributyrin). The beverages prepared using a low LCT to SCT ratio (0:100) were physically unstable mainly due to Ostwald ripening phenomena, as indicated by confocal laser microscopy. The oil droplet size was significantly reduced for emulsions formulated with corn oil (2.6 μm) compared with tributyrin (5.4 μm). Lycopene was not bioaccessible in beverages formulated with tributyrin only and bioaccessibility increased significantly with increasing the LCT to SCT ratio. Data indicated that bioaccessibility for lycopene is 2.7% for emulsions with high LCT ratios (>75). Results indicate that the carrier lipid phase of emulsion-based systems is critical for the formulation of functional drinks for the delivery of lipophilic bioactive compounds.

Keywords: Beverage emulsion; Lycopene; Corn; Tributyrin; *In vitro* digestion; Bioaccessibility

Introduction

Lycopene is a natural carotenoid pigment responsible for the colour of tomato and other red fruits and vegetables, such as papaya, guava, watermelon and grapefruit (Shi & Maguer, 2000). Lycopene may have a beneficial role in chronic disease prevention, which is mainly attributed to the ability of the carotenoid to inactivate reactive oxygen species and delay oxidative

damage. For instance, both *in vitro* and *in vivo* studies, demonstrate its preventive effect against atherosclerosis and other conditions linked to cardiovascular disease (Xaplanteris *et al.*, 2012). In addition, lycopene may also be effective for cancer prevention, thanks to its ability to reduce oxidative stress, inhibit cell proliferation and increase apoptosis of human cancer cell lines (Tapiero *et al.*, 2004). Epidemiological studies suggest that high plasma levels of lycopene is inversely correlated to the risk of bladder, prostate and breast cancer (Heber & Liu, 2002). While no ideal daily dosage has been established for lycopene, consumption of 10-30 mg of lycopene daily is considered adequate to support optimal health (Devaraj *et al.*, 2008).

As new scientific evidence emerges supporting the benefits of lycopene for chronic disease prevention, there is increasing consumer interest for foods enriched with this natural pigment. Currently, the main lycopene dietary sources are tomatoes and tomato-based products (i.e. sauces, juice, purees etc.). The bioaccessibility of lycopene from fresh tomato products is considered low (0.1%-3%) due to its lipophilic nature and its subcellular compartmentalisation within chromoplasts (Salvia-Trujillo & McClements, 2016). Furthermore, lycopene contains 11 double bonds in its structure and as a result is a highly unsaturated carotenoid, which makes it susceptible to degradation via isomerization and oxidation during processing and storage (Shi, 2000). For these reasons, although the addition of lycopene to food products is strongly desirable, an effective formulation strategy in combination with effective processing methods are required to increase the amount of lycopene released from the food matrix (Reboul *et al.*, 2006). Furthermore, a very limited number of studies are available which investigate the bioaccessibility of lycopene powder extract in complex food systems.

Emulsion-based systems are becoming increasingly popular food matrices for encapsulating and delivering hydrophobic compounds with health promoting properties. This is mainly due to the fact that lycopene's bioaccessibility significantly improves in the presence of oil, because free fatty acids enhance the solubilisation efficacy of the carotenoid into mixed micelles and

small vesicles during the digestion process (Colle *et al.*, 2012; Yao *et al.*, 2014). Moreover, previous studies indicate that the different length of the fatty acid chain has an impact on the bioaccessibility of lipophilic bioactive compounds. Several *in vitro* studies suggest that the bioaccessibility of hydrophobic components is lower in emulsion-based systems formulated with SCT compared with the ones containing MCT and LCT (Ahmed *et al.*, 2012; Qian *et al.*, 2012; Salvia-Trujillo *et al.*, 2013). The reasons for this outcome may be attributed to the different products formed during lipid digestion. Thus, the formulation and processing steps of the manufacturing process need to be carefully designed to ensure optimum bioavailability of the bioactive ingredient from an emulsion-based system (Raikos & Ranawana, 2017).

The long-term chemical and physical stability of orange oil beverage emulsions formulated with LCT and SCT, which contain lycopene as a bioactive ingredient, has been recently documented (Meroni and Raikos, 2018). The primary focus of the present study was the design of an emulsion-based system that can be used to enhance the bioaccessibility of lycopene and to clarify the specific effect of fatty acid chain length to this respect. Beverage emulsions were prepared from carrier lipids that consisted of different LCT-to-SCT (w/w) mass ratios as follows: 100:0, 75:25, 50:50, 25:75 and 0:100. The effect of different oil composition was evaluated by studying the following parameters: (1) physical stability of freshly prepared beverages monitored by visual observation, Turbiscan and microstructural analysis; and (2) bioaccessibility of lycopene by using an *in vitro* gastro-intestinal digestion model. Results of this study have important implications for developing effective emulsion-delivery systems of lycopene in order to meet consumer demands for food products with health-promoting properties.

Materials and methods

Materials

Lycopene powder (lycopene>10%, redivivo®) was kindly provided by DSM Nutritional Products Ltd (Heanor, UK). Tocopherol-stripped corn oil (LCT) and tributyrin (SCT) were purchased from Sigma–Aldrich (Dorset, UK). Citric acid, amylase (type VI-B), pepsin, pancreatin, bile extract and Nile Red were purchased from Sigma Aldrich (Dorset, UK). Pure Whey Isolate™ 97 powder (WPI, 97% protein) was purchased from Bulk Powders (Colchester, UK). All other reagents used were of analytical grade.

Determination of Total Lipids as Fatty Acid Methyl Esters (FAME)

The fatty acid composition is determined as the methyl esters of fatty acids by a Hewlett Packard 6890 gas-liquid chromatograph (Avondale, PA) equipped with a 50 m × 20 mm Chrompac CP7488 CP Sil-88 capillary column (film thickness 0.20 µm). The experimental procedure is described in detail in literature published previously (Meroni & Raikos, 2018). Separation was recorded with HP GC Chemstation software (Hewlett Packard, Avondale, PA). Results are expressed as % of total fatty acids.

Preparation of oil-in-water (o/w) beverage emulsions

Emulsion beverages were obtained by mixing the following ingredients using a standardized (w/w) recipe: 90.3% water, 3% WPI, 3% oil, 0.7% citric acid, 1% lycopene powder. To investigate the effect of fatty acid chain length, emulsions were prepared from carrier lipids that consisted of different mass ratios of LCT-to-SCT (w/w) as follows: 100:0, 75:25, 50:50, 25:75 and 0:100. A coarse emulsion was initially formed by slowly adding oil to the water phase and mixing the rest of the ingredients using an ultra-compact digital mixer system (Cole-Palmer, Cambridgeshire, UK) for 5 min at 1000 rpm. Emulsions were formed by passing the coarse emulsions twice through a single stage valve homogenizer (APV-1000, SPX Flow Technology, West Sussex, UK) at 50 MPa. The process was repeated twice to generate two

different batches for every oil type beverage. Physical stability analysis was performed immediately after emulsion formation. For other analyses samples were stored at 4 °C for up to one week after emulsion formation. The method of preparation is described in detail in previous work (Meroni & Raikos, 2018).

Emulsion physical stability

A Multiple Light Scattering method (Mengual *et al.*, 1999) was employed to assess the physical stability of beverage emulsions using a Turbiscan MA2000 (Formulaction, Ramonville St. Agne, France). The apparatus can measure backscattered light as a function of the distance along the axis of the sample container and time by using a synchronous optical sensor that receives light backscattered by the sample. Backscattered flux is directly related to the photon transport mean free path (l^*).

$$BS = \frac{1}{\sqrt{l^*}} \quad (1)$$

Mie theory states that l^* is inversely proportional to the volume fraction of samples and proportional to the mean diameter d , which is represented by the equation:

$$l^*(\Phi, d) = \frac{2d}{3\Phi(1-g)Q_s} \quad (2)$$

where d is the average particle diameter, Φ is the volume fraction occupied by particles, g is the asymmetry factor and Q_s is the extinction cross-section divided by the geometrical cross-section. Quantification of creaming was enabled by calculating the particle migration velocity and the thickness of the cream phase. A series of scans was repeated for the beverages at 5 min intervals from top to bottom and the intensity of light backscattered or transmitted during a 1 h period at 37°C was recorded. The refractive indices of the dispersed and continuous phase which were used to compute the mean spherical equivalent diameter were 1.45 and 1.33 respectively. Turbisoft Lab 2.2 software was used to monitor the destabilization phenomena through the variation of the backscattering flux over time.

Transparency analysis

Transparency of lycopene beverage emulsions was acquired by measuring the absorbance of diluted beverage samples (x200) at 600 nm by means of A Pye Unicam UV-4 UV-VIS scanning spectrophotometer (Spectronic Camspec Ltd, Leeds, UK) according to the method described by Ha *et al.* (2015). The following equation was used to calculate transparency:

$$T = \frac{1}{10^A} \quad (3)$$

where T is the transparency and A is the value of absorbance at 600 nm.

Emulsion microstructure

Emulsion microstructure was analyzed by means of confocal laser scanning microscopy (CLSM) using a Carl Zeiss LSM 710 (Carl Zeiss Ltd, Cambridge, UK) according to the method of Chevallier *et al.* (2016). In brief, 10 µl Nile Red solution (0.125% w/v in propylene glycol) were mixed with 1 ml of emulsion to dye the fat globules. Samples were kept at 25 °C in the dark for 15 min, then 50µl of each emulsion were placed on microscope cover glass. Observations were conducted at excitation wavelength of 543 nm for Nile Red dye and using a 63x oil immersion objective. Images for each sample were captured by scanning at a resolution of 1024x1024 pixels.

In vitro gastrointestinal digestion method (IGD)

The method described by Minekus *et al.* (2014) based on the standardized static *in vitro* digestion model suitable for food was used to determine lycopene bioaccessibility with several modifications (Meroni & Raikos, 2018). The compositions of the simulated digestion fluids for the oral, gastric and intestinal phases are presented in Table 1. Four independent IGD runs were performed to obtain replicates and assess reproducibility of results.

Quantification of lycopene

Lycopene in beverages and digested samples was quantified by means of a reverse phase HPLC method using fluorescence and UV-visible detection according to the method of Hess *et al.* (1991) with modifications (Meroni & Raikos, 2018). Peaks were identified by comparing the retention times and UV-Vis spectral data with those of the corresponding standards and quantification was enabled by using the standard curves. Measurements were determined with mixed standards containing carotenoids and tocopherols at appropriate concentrations and results were expressed in µg/g of oil. Echinone was added as an internal standard for accurate quantitative measurement.

Bioaccessibility determination

Following the digestion protocol, the digesta was centrifuged (3220 x g) at 25 °C for 40 min using a MiniSpin® plus centrifuge (Fisher Scientific UK, Loughborough, UK). The middle phase of the centrifuged sample was assumed to contain solubilized lycopene in mixed micelles (Ha *et al.*, 2015). Aliquots were collected directly from raw digesta and from the middle phase of centrifuged samples and were prepared for RP-HPLC analysis for lycopene quantification. The following equation was used to determine bioaccessibility of lycopene:

$$\text{Lycopene bioaccessibility (\%)} = 100 \times \frac{C_{\text{micelle}}}{C_{\text{initial sample}}} \quad (4)$$

where C_{micelle} is the lycopene concentration in the mixed micelle phase after *in vitro* digestion.

Statistical analysis

All experiments were conducted on at least two freshly prepared beverages. Results are expressed as means ± standard deviation (SD) of at least three replicates. Data were subjected to statistical analysis by SPSS Statistics 22 software. Means were analyzed by analysis of

variance (ANOVA) and significant differences ($p < 0.05$) were detected by the *Scheffé's* post hoc test.

Results and discussion

Effect of carrier oil on physical stability and optical properties of beverage emulsions

A series of oil-in-water emulsions was produced by high pressure homogenization that contained different ratios of LCT (corn oil) and SCT (tributyrin). Tributyrin (SCT), is a common food additive composed of butyric acid and is naturally present in butter. Corn oil (LCT) contains a desirable fatty acid profile and is a key ingredient in many processed foods. The detailed fatty acid composition of each carrier oil is in agreement with published data (Table 2). As expected the analysis of the oils confirmed that corn oil is a good source of long chain triglycerides, predominantly linoleic acid, oleic acid and palmitic acid. Tributyrin is a saturated short chain triglyceride composed of butyric acid esterified to glycerol.

The physical stability of the beverage emulsions was monitored by a. visual observation, b. Turbiscan analysis and c. microstructural analysis. All methods employed in this study, indicated that the fatty acid composition had a major impact on the beverage emulsions' stability and their susceptibility to instability phenomena. Figure 1 shows the beverages standing at room temperature for 24 h. Visual observation of the samples suggested that beverages with a high corn oil concentration were prone to creaming as evidenced by the formation of a cream layer at the top of the emulsion. On the other hand, beverages containing 100% tributyrin were prone to sedimentation phenomena with the droplets being visually detected at the bottom of the emulsion. These differences in the direction of the migration pattern between samples with high levels of corn oil and tributyrin are governed by their corresponding densities (0.91 g/ml & 1.03 g/ml at 25 °C respectively).

A more detailed insight into the physical stability of the beverage emulsions is given by Turbiscan analysis, which enables the early detection of instability phenomena by evaluating the optical transmission and backscattering profiles of undiluted emulsion samples. Results obtained from Turbiscan analysis are presented in Table 3. Increasing the LCT/SCT ratio of the beverage led to significant ($p < 0.05$) reduction of the particle size. The beverages formulated with tributyrin (100%) have an average particle size droplet more than twice as large compared with samples formulated with corn oil (100%). Results are attributed to Ostwald ripening (OR) phenomena which are commonly observed in food systems containing emulsified tributyrin (Wooster *et al.*, 2008). Ostwald ripening is the process by which the components of the discontinuous phase diffuse from small to large droplets through the continuous aqueous phase (Kabalnov & Shchulkin, 1992). Data from microstructural analysis of the beverages also indicates that OR is the main cause of instability for the samples formulated with 100% tributyrin (Figure 2). Ostwald ripening rates are directly proportional to oil molar volume (Wooster *et al.*, 2008). **Emulsions formulated with tributyrin as the sole lipid phase are highly unstable to droplet growth due to Ostwald ripening (OR) because of the relatively high water solubility of this low molecular weight triacylglycerol** (Li *et al.*, 2009). The addition of corn oil enhances the physical stability of the beverages by significantly reducing the average droplet particle size and this effect is more profound with increasing the LCT ratio of the mixture (Figure 2). Previous research has indicated that droplet enlargement due to OR can be greatly reduced by the addition of highly hydrophobic triglycerides such as corn oil (McClements *et al.*, 2012). The enhanced stability against OR when LCT are added to tributyrin is attributed to the altered lipid phase composition between differently size droplets, a phenomenon known as compositional ripening. The large triacylglycerol molecules of corn oil with low water-solubility prevents droplet growth by a ripening effect that opposes OR effect (Kabalnov & Shchulkin, 1992). On the other hand creaming rates are higher for samples

with a high LCT content, which is in agreement with the visual observations of the beverage emulsions during storage at 25 °C for 24 h. Thus, although corn oil is essential to inhibit OR and stabilize beverage emulsions which contain SCT, it is not ideal for formulations as the sole lipid carrier due to creaming effects. Results suggest that a combination of corn oil and tributyrin (75:25) is desirable to obtain favorable physical stability of beverage emulsions.

Optical properties of emulsion-based products are of paramount importance and relate to consumer liking and product acceptability. The interplay between nutrient composition, emulsion microstructure and appearance needs to be elucidated for designing emulsion-based products with desirable characteristics. The appearance of emulsions to the human eye is determined by interactions with electromagnetic radiation in the visible region of the spectrum. The colour of an emulsion is determined by the presence of chromophoric compounds. Light scattering effects are primarily dependent by the characteristics of emulsion droplets such as size, concentration, and refractive index (Park *et al.*, 2013). Figure 3 shows the relationship between particle size, LCT-SCT ratio and (%) transparency. Results indicated that increasing particle size increased transparency. The findings of this study are not in agreement with previous research which suggests that small particle size corresponds to higher transparency of nanoemulsions (Ha *et al.*, 2012). At nanoscale the optical properties are largely influenced by the particle size of the dispersed particles. For macroemulsions, the refractive indices of the oil and water phase play a major role in emulsion transparency. If both phases have the same or similar refractive index (n), there will be neither reflection nor refraction and the system will appear homogeneous and entirely transparent (Poras *et al.*, 2008). In the present study, the refractive index difference between oil and aqueous phase is higher for beverages with increasing LCT (water: 1.33, corn: 1.47, tributyrin: 1.43), which seems to be the main reason for the improved transparency shown by the beverages with a high SCT/LCT ratio.

278 Impact of carrier oil on bioaccessibility of lycopene during *in vitro* gastric digestion of
279 beverage emulsions

280 The term bioaccessibility refers to the fraction of a nutrient or compound that can be absorbed
281 in the small intestine after it has been released from the food matrix during digestion. A number
282 of studies indicate that the addition of dietary fat is beneficial for increasing the bioaccessibility
283 of carotenoids (Brown *et al.*, 2004; González-Casado *et al.*, 2018). In this study, five ratios
284 from two oil types were chosen to assess the effect of fatty acid length and saturation degree
285 on lycopene bioaccessibility from a beverage emulsion using a simulated gastrointestinal
286 digestion procedure. Bioaccessibility was determined by taking into account the lycopene
287 concentration in the mixed micelle phase and in the undigested sample. This calculation
288 method quantifies the lycopene fraction which is made available for absorption in relation to
289 the amount originally present in the beverage, and is thus more indicative of the amount of the
290 carotenoid lost during the digestion process. Results clearly indicated that lycopene
291 bioaccessibility was significantly affected by the type of carrier oil used for emulsion
292 formulation (Fig. 4). In particular, the highest bioaccessibility value for lycopene was recorded
293 for the beverage formulated with an LCT-to-SCT ratio of 75:25, whereas no lycopene was
294 available for absorption from the samples formulated with tributyrin only. In fact, lycopene
295 bioaccessibility increased with increasing the amount of LCT present in the oil phase of the
296 emulsion. The present findings are in agreement with previously published data, which further
297 confirm the hypothesis that bioaccessibility of carotenoids depends on the type of carrier oil
298 used for emulsion formulation. In a similar study, bioaccessibility of β -carotene encapsulated
299 in nanoemulsions was highly affected by the carrier oil type and decreased in the order
300 LCT>MCT>undigested oil (Qian *et al.*, 2012). Moreover, Salvia-Trujillo *et al.* (2013) reported
301 that β -carotene bioaccessibility in edible nanoemulsions increased from 14% to 86% as the
302 LCT fraction of the oil phase increased from 0% to 100%. Similarly, emulsions containing

MCT or LCT within the carrier lipid were able to substantially increase the bioaccessibility of curcumin, a pigment from turmeric, which can be attributed to their ability to form mixed micelles capable of solubilizing components of lipophilic nature (Ahmed *et al.*, 2012). LCT from corn oil are capable of forming mixed micelles with a large hydrophobic core, whereas those produced by digestion of SCT, do not form mixed micelles that are capable of solubilizing large, hydrophobic carotenoids (Fatouros & Mullertz, 2008). Thereby, the colloidal structures formed by the digestion of LCT allow the accommodation of lycopene, which in turn increases the solubilisation capacity of the carotenoid prior to absorption. This mechanism is proposed in several papers (Rao *et al.*, 2013; Qian *et al.*, 2012; Salvia-Trujillo *et al.*, 2013) and may explain why lycopene bioaccessibility was significantly higher for beverages with increasing LCT-to-SCT ratios. However, this hypothesis needs to be further investigated to confirm whether LCT can enhance the absorption of lycopene *in vivo*.

An additional factor that has been previously reported to affect lycopene bioaccessibility is the size of the fat globules present in the emulsions. Emulsions with small droplets have large specific surface area that increases the accessibility to lipases, co-lipases, and endogenous surfactants (bile salt, cholesterol, phospholipids); consequently the digestion rate, solubilisation efficiency and the absorption rate of the encapsulated compound is enhanced by reducing the droplet size. The first study to report the direct correlation between oil droplet size and bioaccessibility of lycopene encapsulated in oil-in-water nanoemulsions was published by Ha *et al.* (2015). A further study showed that the droplet size of excipient emulsions can determine the bioaccessibility of lycopene from tomato juice (Salvia-Trujillo & McClements, 2016). In this study, the bioaccessibility of lycopene increased from 10% to 12.5% and this effect was attributed to the reduction of size of the oil globules. This resulted in higher exposure of lipid surface area, which in turn enhanced the digestion rate and efficiency by digestive enzymes. Salvia-Trujillo *et al.*, 2013). These results are in agreement with the findings of the

present study, which show an inverse correlation between droplet size and bioaccessibility; the highest lycopene bioaccessibility (2.7%) was obtained for the beverages with the highest LCT-to-SCT ratio (100:0) and the smallest droplet size ($2.6\mu\text{m}\pm 0.1$). These results are consistent with the previously reported data (Ha *et al.*, 2015; Salvia-Trujillo & McClements, 2016; Salvia-Trujillo *et al.*, 2017), and confirm the importance of droplet size for the bioaccessibility and absorption of lycopene from emulsion-based systems.

Conclusions

This study shows that the ratio of long- to short-chain triglycerides of the dispersed oil phase has a significant impact on the physical stability of beverage emulsions. The addition of corn oil significantly reduced the average droplet size and enhanced the physical stability of the beverages by retarding Ostwald ripening phenomena; this effect was more profound with increasing the LCT ratio of the mixture. Nevertheless, emulsions with high LCT content had higher creaming rates and lower transparency compared to the beverages with high SCT-to-LCT ratios. The bioaccessibility of lycopene was significantly increased with the inclusion of LCT in the beverage formulation, whereas it was not bioaccessible in beverages containing tribyturin only. Both droplet size and the solubilisation efficiency of LCT's impact on lycopene bioaccessibility from beverage emulsions.

Considering the results obtained in this study, we can conclude that the most favorable formulation for beverage emulsions containing lycopene should contain a mixture of LCT and SCT, at a ratio 75:25. Findings of this research highlight the importance of the selection of carrier oil for designing emulsion delivery systems capable of encapsulating lycopene. Further improvements with respect to lycopene formulation in complex food systems are required and future studies to clarify the effect of different oil types on the bioavailability of lycopene using *in vivo* models (cells, animals or humans) are needed.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Legends to Figure

Fig. 1. Effect of LCT to SCT ratio on the particle migration pattern by visual observation at room temperature for 24 hr.

Fig. 2. Confocal Laser Scanning Microscopic (CLSM) images of beverage emulsions with different LCT to SCT ratios. Lipid droplets are stained with Nile red and scale is scale bar equals to 10 μm .

Fig. 3. Relationship between transparency (%), LCT to SCT ratio and particle size (μm) of lycopene beverage emulsions.

Fig. 4. Bioaccessibility (%) of lycopene beverage emulsions formulated with different LCT to SCT ratios. The centrifuged fraction of the raw digesta (mixed micelles) of the beverage samples is shown in the picture. Different letters denote significant differences ($P < 0.05$) between samples.